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(54) **Assay for cholesterol and derivatives thereof.**

(57) A cholesterol sensor is a dry strip sensor which comprises an elongate substrate (I) with a screen printed reference electrode (6) and a screen printed working electrode (5), the working electrode comprising a conducting surface carrying optional cholesterol esterase, a cholesterol oxidase, a peroxidase, and a mediator which transfers charge between the conducting surface and the peroxidase when the peroxide is catalytically active.

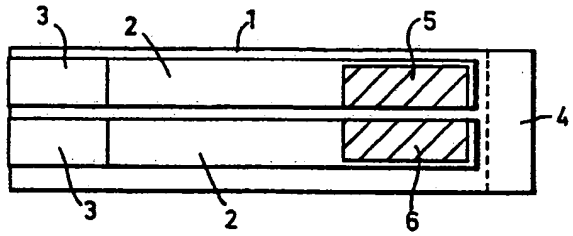

FIG.1.

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ASSAY FOR CHOLESTEROL AND DERIVATIVES THEREOF

The present invention relates to an assay for free or total cholesterol in blood.

BACKGROUND OF THE INVENTION

Elevated levels of cholesterol and its esters are recognised as risk factors for arteriosclerosis and myocardial infarction. The normal range of serum values extends from 3 to 6 mM for total cholesterol (free cholesterol and cholesterol ester), while in hyperlipidaemic conditions the level can increase to 10 mM and above.

Procedures for cholesterol determination have employed a variety of chemical and physical techniques. Traditional assays involve chemical treatment of cholesterol to yield coloured products which can be measured spectrophotometrically. The most common reactions are the Liebermann-Burchard reaction, the iron salt-sulphuric acid reaction. and the p-toluene sulphonic acid reaction. However, these various traditional procedures involve numerous drawbacks, including a lack of specificity, difficulty in standardization, the use of unstable and corrosive reagents, a variable reactivity of the esters, and interference by other molecules such as haemoglobin and bilirubin.

Most modern methods for blood cholesterol determination are enzymic, and take advantage of the specificity afforded by enzymes. Typically the detection is spectrophotometric. A chromogen is generated through a series of stoichiometrically coupled reactions, such as;

(A): cholesterol ester + H_2O = cholesterol + fatty acid

(B): cholesterol + O_2 = cholestenone + H_2O_2

(C): H_2O_2 + phenol + 4-aminophenazone = 4-(p-benzoquinone-monoimino)-phenazone + $4H_2O$

The reactions (A), (B), and (C) are respectively catalysed by a cholesterol esterase, a cholesterol oxidase, and a peroxidase.

The phenazone derivative formed in reaction - (C) has specific spectroscopic properties and therefore the cholesterol concentration in a sample can be determined by the amount of the phenazone derivative produced under appropriate conditions.

The disadvantages of such methods include the particular difficulties of calibration when blood plasma is being assayed, especially where the turbidity of the plasma must be taken into consideration. Moreover, this method is not suitable for assay of cholesterol in whole blood and consequently an expensive and time-consuming preparation of the sample must be carried out.

Other techniques may be employed for determination of the products of enzymic assays for cholesterol. For example, European Patent Application 84303085, published as EPI25867, describes a method of assay in which an electrode poised at a suitable potential is contacted with a system comprising a first enzyme, a cofactor linked with said enzyme and a mediator compound which transfers charge to the electrode from the first enzyme when its electrical state is changed by reaction of cofactor material. The cofactor may be NAD, NADP, cAMP, ATP, GTP, TTP, or CTP.

In one example of a procedure in accordance with EPI25867, an electrochemical determination of cholesterol is performed by oxidizing cholesterol to cholestenone using cholesterol dehydrogenase in the presence of NAD as cofactor, followed by oxidation of the resultant NADH with a diaphorase whose catalytic activity is detected at an electrode using a ferrocene compound as a mediator.

In practice, there are some difficulties in adapting such a detection procedure for use with blood samples. Specifically, it seems that the enzymes are extremely sensitive to inhibition by surfactants of the kind conventionally employed to release cholesterol esters from the lipoprotein complex in whole sera.

OBJECTS OF THE INVENTION

It is an object of this invention to provide an assay which can be performed rapidly, accurately and at low cost by persons such as general medical practioners in their own surgery. It is a further object of the present invention to provide sensors for such an assay. A specific object of this invention is an assay which can be performed without the need for elaborate preliminary treatment of a blood sample.

SUMMARY OF THE INVENTION

The present invention provides a sensor for use in the assay of cholesterol in blood. The sensor is a dry strip sensor, and comprises an elongate substrate with a screen printed reference electrode and a screen printed working electrode. The working electrode comprises a conducting surface, preferably of finely divided carbon optionally with a

silver tracking, carrying a reagent mix. The reagent mix includes a surfactant, a cholesterol oxidase, a peroxidase, and a mediator which transfers electrons between the conducting surface and the peroxidase in the presence of peroxide as substrate. The mix further includes a cholesterol esterase if assay of total cholesterol is desired.

Without being bound by theory, it is believed that in an assay in accordance with the present invention, the surfactant serves to break up the lipoprotein complex of blood, and cholesterol is then oxidised to cholestenone by the cholesterol oxidase. Such oxidation is accompanied by oxygen to hydrogen peroxide which is then itself further reduced to water by the peroxidase. The catalytic activity of the peroxidase is detected at an electrode using the mediator to transfer electrons. If the optional cholesterol esterase is present, then cholesterol esters are first converted to cholesterol, which will not occur if the assay is only for free cholesterol and the esterase is omitted.

It seems that in the present assay, the mediator is electrochemically reduced at the electrode, producing a current measurable at the electrode, which current is related to the activity of the cholesterol oxidase and hence the amount of cholesterol present in the sample.

FEATURES OF THE INVENTION

It is a feature of this invention that the sensor is a dry strip sensor. Surprisingly, it was found that a similar mix of reagents employed in a wet sensor system did not give good results across a desired range of detectable cholesterol concentrations.

It is a further feature of this invention that the dry strip sensor is produced with the aid of screen printing. Given that the reagent mix includes enzymes, it will be implicit that a low temperature printing procedure is required.

The mediator compound may be selected from ferrocyanide. ruthenium compounds, carboranes, polyviologens, one-dimensional conductive salts of TCNQ, haloanils and derivatives thereof, alkyl substituted phenazine derivatives, and, bis-cyclopentadienyl $(Cp)_2MX_x$ complexes including ferrocene and its derivatives. The mediator is preferably ferrocene or a derivative thereof, especially a water-soluble ferrocene derivative.

In a particularly preferred embodiment, the dry strip sensor of this invention comprises an elongate, electrically-insulating substrate having a pair of longitudinal, substantially parallel, electrically-conducting tracks thereupon, each track being provided at the same end thereof with means for electrical connection with read-out means and provided at the other end thereof with an electrode, one of the electrodes being the reference electrode and the other being the working electrode responsive to the presence of cholesterol or its esters.

In performing an assay, the working electrode is preferably fixed at a small negative voltage relative to an Ag/AgCl reference counter-electrode.

The present invention will be further illustrated by way of example and with reference to the accompanying drawings.

SUMMARY OF THE DRAWINGS

In the drawings,

Figure I shows a screen-printed sensor according to the present invention,

Figure 2 shows a graph of hydrogen peroxide concentration against current in μA, for a sensor according to the present invention,

Figure 3 shows a graph of cholesterol concentration against current in μA, for a sensor according to the present invention,

Figure 4 shows a graph of cholesterol ester concentration against current in μA, for a sensor according to the present invention, and

Figures 5 and 6 show comparative results obtained with wet systems not in accordance with the present invention.

EXAMPLES OF THE INVENTION

Example I: construction of dry strip sensor

Commercially obtained horse radish peroxidase, cholesterol oxidase, and cholesterol esterase were reconstituted in an imidazole buffer mix of pH 7 for 4 hours prior to the addition of finely divided carbon. The working electrode was then screen-printed onto a preprinted carbon track laid down upon a PVC substrate. The mix was allowed to dry.

The screen-printing process may comprise the following steps;

a) printing of a conductive tracking,
b) printing of a working electrode,
c) printing of a reference electrode, and,
d) printing of a dielectric insulation.

Figure I shows a PVC sheet (I) which comprises the supporting substrate for the electrode. Conductive carbon tracking (2) is screen-printed onto the surface of the substrate in two conductive tracks of 45mm by 2mm, with a separation of 3mm. These tracks are overlaid with silver conductive ink to form contacts (3) for connection of the sensor to readout apparatus.

The working electrode (5) and the reference counter-electrode (6) are applied to the ends of the tracking (2) by screen printing. The Ag/AgCl reference electrode (6) employed in the present instance is a quasi-reference electrode which adopts a potential determined by a redox couple added to the reference electrode in the dry phase.

If desired, a mesh can be applied over the printed electrodes to give protection. The mesh can be coated with one or more of the electrode reagents.

In the present example, the tracks (2) and the conductive silver ink do not extend the complete length of the substrate (1), facilitating manipulation of the sensor and insertion into readout apparatus.

Example 2: calibration of electrodes.

A sample of the species to be determined in buffer, blood or serum is then applied to a target area covering both the working electrode and the reference counter-electrode. Cabibration curves were calculated for the three analytes, cholesterol ester, cholesterol or H_2O_2.

During calibration the potential was poised at -50mV (vsSCE). Fixed potential studies employing a range of hydrogen peroxide concentrations (0-10mM) in imidazole (pH 7.0) buffer and in serum were carried out. A linear steady-state current response with respect to hydrogen peroxide concentration was recorded between 0.1mM and 10mM hydrogen peroxide. The results of one such calibration experiment are shown in figure 2, in which hydrogen peroxide concentration is plotted against current in μA, for a sensor according to the present invention.

Example 3: cholesterol detection

A study similar to that described in Example 2 was carried out using cholesterol standards (Sigma Chemical Company). Steady state currents were obtained proportional to the cholesterol concentration giving a linear relationship from 1mM to 10mM. The results of one such calibration experiment are shown in figure 3, in which cholesterol concentration is plotted against current in μA, for a sensor according to the present invention.

Example 4: cholesterol ester detection.

A range of samples with varying cholesterol ester concentrations was made up using a formulation containing a surfactant. The nature of the surfactant is not critical, and suitable trial will establish if a candidate surfactant gives adequate breakdown of the blood lipoprotein complex. It was found possible to make up 10mM cholesterol ester in 10% surfactant.

The samples were applied to the electrode target area and allowed to incubate for four minutes at a poised potential of 50mV vs SCE. Current values proportional to the cholesterol ester concentrations were obtained. The results of such an experiment are shown in figure 4, in which cholesterol ester concentration is plotted against current in μA, for a sensor according to the present invention.

Comparative Example: a wet system

An electrochemical glass cell with a working volume of 1 ml was set up with a gold working electrode and a platinum gauze as counter electrode. A mix of assay reagents was made up to 0.7 ml, and transferred to the cell. After appropriate poising of the working electrode, the current response was monitored on additon of the enzyme.

In a first experiment, for which the results are shown in Figure 5, the mediator was ferrocene methylamine hydrochloride and the working electrode potential was +105 mV. For a second experiment, Figure 6, ferrocene monocarboxylic acid was employed with a working electrode potential of +44 mV and varying concentrations of cholesterol oxidase.

It will be seen that a non-linear response was obtained for each of these wet systems. In further experiments, it was established that the use of the dry strip sensor was essential to the present invention.

Claims

1. A sensor for use in the assay of total cholesterol in blood, the sensor being a dry strip sensor comprising an elongate substrate with a screen printed reference electrode and a screen printed working electrode, the working electrode comprising a conducting surface carrying a surfactant, a cholesterol esterase, a cholesterol oxidase, a peroxidase, and a mediator which transfers electrons between the conducting surface and the peroxidase in the presence of peroxide as substrate.

2. The sensor of claim 1, which comprises an elongate, electrically-insulating substrate having a pair of longitudinal, substantially parallel, electrically-conducting tracks thereupon, each track being provided at the same end thereof with means for electrical connection with read-out means and provided at the other end thereof with an electrode,

one of the electrodes being the reference electrode and the other being the working electrode responsive to the presence of cholesterol.

3. A sensor for use in the assay of free cholesterol in blood, the sensor being a dry strip sensor comprising an elongate substrate with a screen printed reference electrode and a screen printed working electrode, the working electrode comprising a conducting surface carrying a surfactant, a cholesterol oxidase, a peroxidase, and a mediator which transfers charge between the conducting surface and the peroxidase in the presence of peroxide as substrate

4. The sensor of claim 3, which comprises an elongate, electrically-insulating substrate having a pair of longitudinal, substantially parallel, electrically-conducting tracks thereupon, each track being provided at the same end thereof with means for electrical connection with read-out means and provided at the other end thereof with an electrode, one of the electrodes being the reference electrode and the other being the working electrode responsive to the presence of cholesterol.

5. An assay for total cholesterol, wherein the cholesterol is amperometrically quantitated using a sensor as defined in claim I.

6. An assay for free cholesterol, wherein the cholesterol is amperometrically quantitated using a sensor as defined in claim 3.

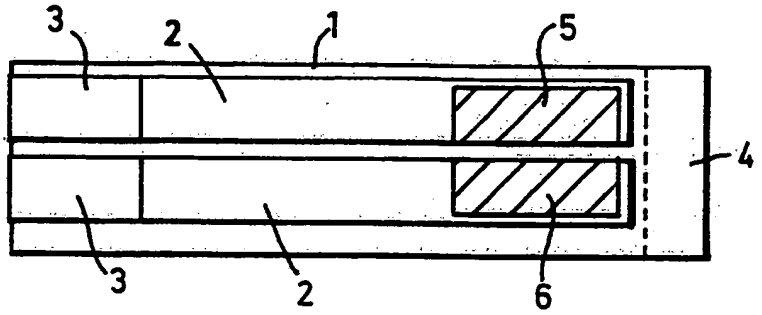
3
2
1
5
4
3
2
6

FIG.1.

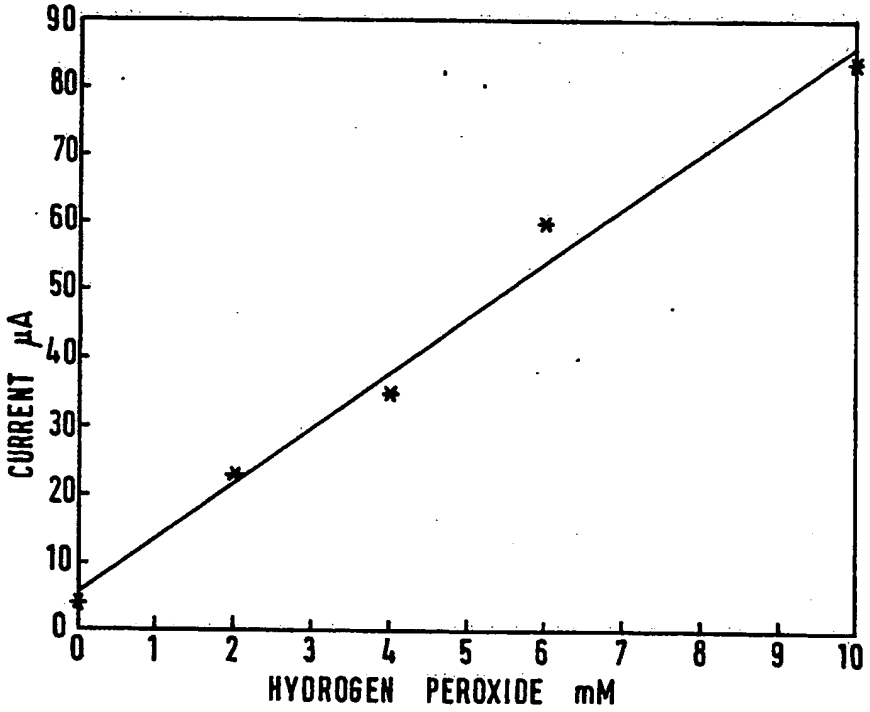
90
80
70
60
50
40
30
20
10
0
CURRENT μA
0
1
2
3
4
5
6
7
8
9
10
HYDROGEN PEROXIDE mM

FIG.2.

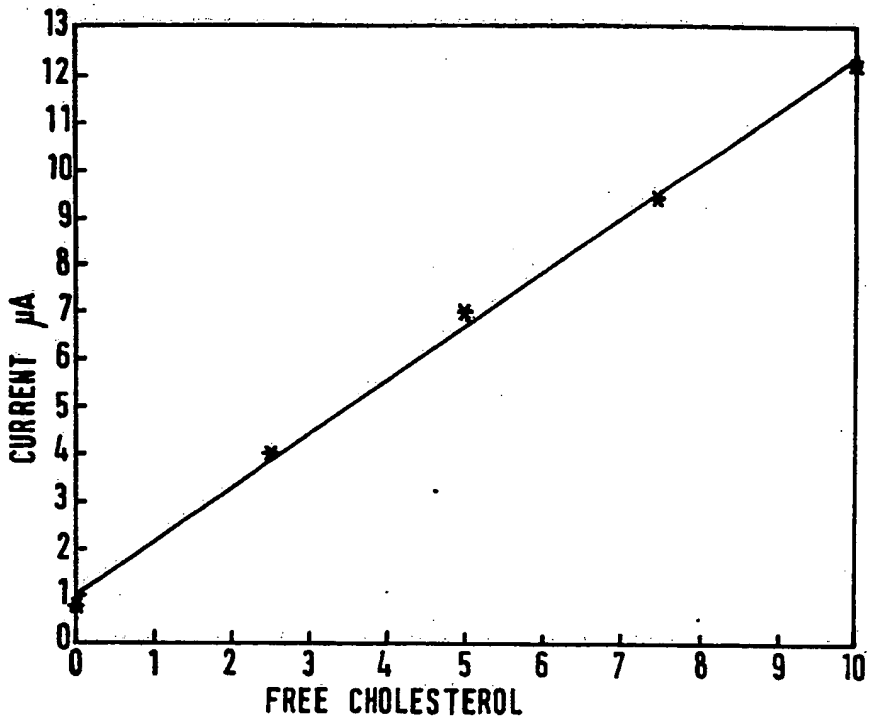
CURRENT µA
FREE CHOLESTEROL
0
1
2
3
4
5
6
7
8
9
10
11
12
13

FIG. 3.

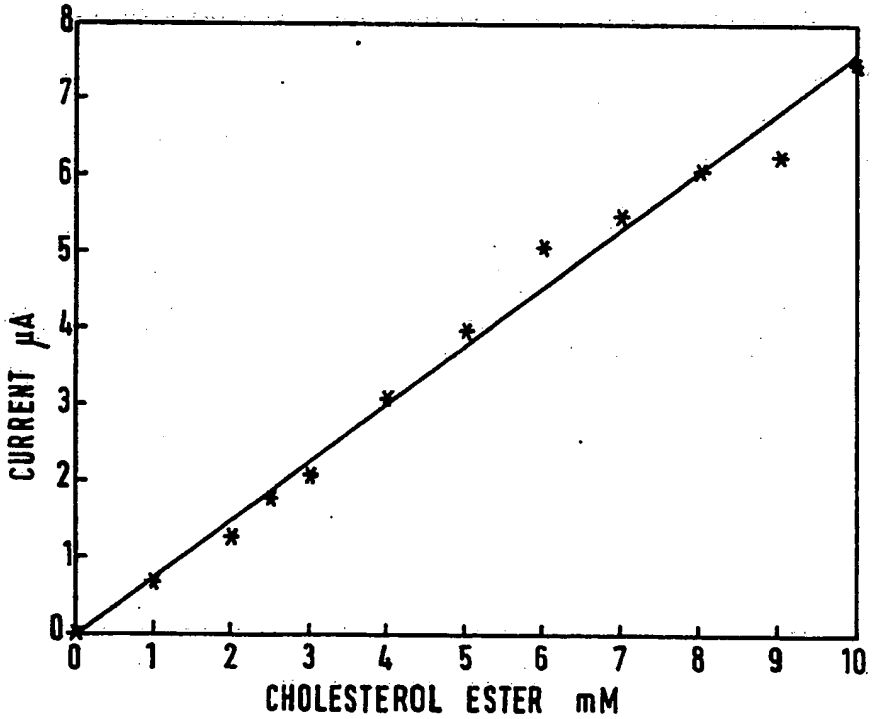
CURRENT µA
CHOLESTEROL ESTER mM
0
1
2
3
4
5
6
7
8
9
10

FIG. 4.

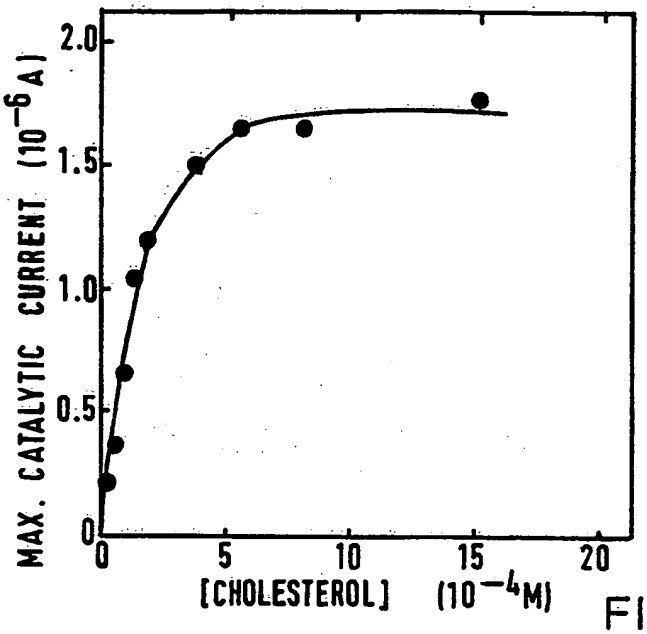
MAX. CATALYTIC CURRENT (10^{-6}A)
2.0
1.5
1.0
0.5
0
0
5
10
15
20
[CHOLESTEROL] (10^{-4}M)

FIG.5.

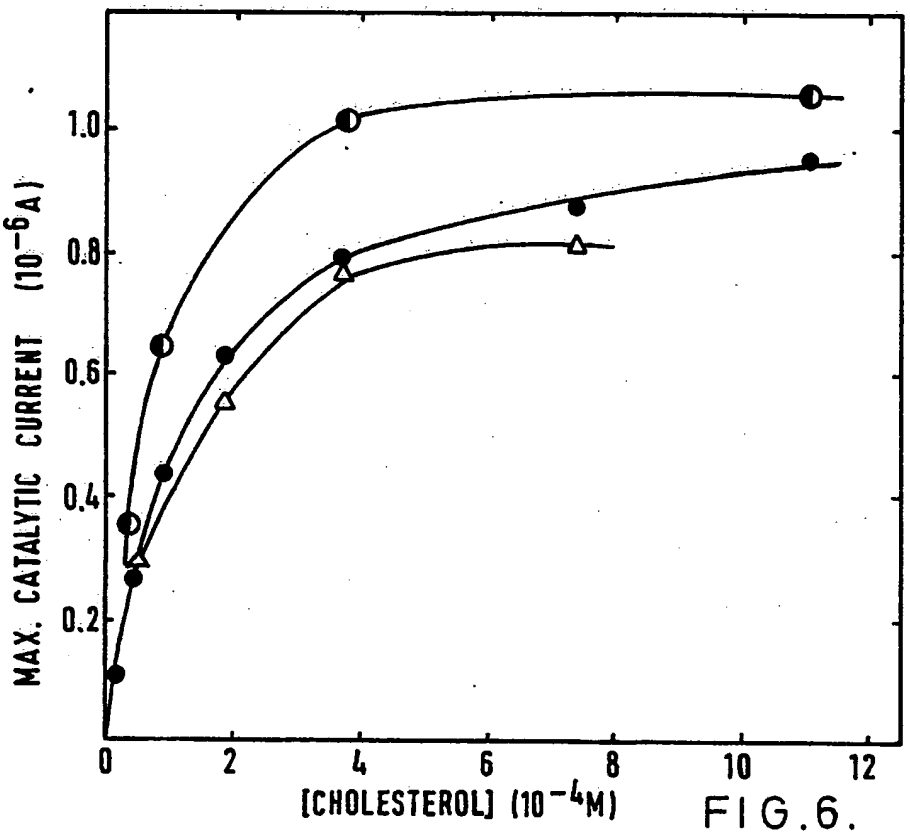
MAX. CATALYTIC CURRENT (10^{-6}A)
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
[CHOLESTEROL] (10^{-4}M)

FIG.6.

European Patent Office

EUROPEAN SEARCH REPORT

Application number

EP 86 31 0151

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	CHEMICAL ABSTRACTS, vol. 101, no. 5, 30th July 1984, page 242, abstract no. 35287e, Columbus, Ohio, US; U. WOLLENBERGER et al.: "Amperometric enzyme sequence electrodes for cholesterol", & BIOELECTROCHEM. BIOENERG. 1983, 11(4-6), 307-317	1-6	C 12 M 1/40 C 12 Q 1/60 G 01 N 33/48
A	CHEMICAL ABSTRACTS, vol. 103, no. 13, 30th September 1985, page 312, abstract no. 101465f, Columbus, Ohio, US; T. YAO et al.: "Amperometric assays of total and free cholesterols in serum by the combined use of immobilized cholesterol esterase and cholesterol oxidase reactors and peroxidase electrode in a flow injection system", & ANAL. BIOCHEM. 1985, 149(2), 387-391	1-6	
A	EP-A-0 121 385 (CAMBRIDGE LIFE SCIENCES PLC) * Whole document *	1-6	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 M G 01 N C 12 Q
A,D	EP-A-0 125 867 (GENETICS INTERNATIONAL INC.) * Whole document *	1-4	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	09-04-1987	HITCHEN C.E.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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